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(57) Abstract

The present invention provides a process for the restoration of a plant phenotype that is altered due to a first transgene which when expressed inhibits expression of an endogenous plant gene, the process comprising introducing into said plant, or progeny thereof, a second transgene which encodes a protein or polypeptide that is capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 90 %.

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Gen tic m derati n r rest rati n f plant phen typ s

FIELD OF THE INVENTION

The present invention relates to genetically transformed plants, methods for obtaining genetically transformed plants and recombinant DNA for use therein. The invention further relates to a method for restoring a plant phenotype previously altered due to the expression of a transgene in that plant.

BACKGROUND ART

The European Patent Application 344 029 A2 describes a method for restoring male-fertility in plants that are male-sterile due to the expression of a first transgene encoding Barnase in the tapetal cell layer of said plants, which method comprises the introduction into the same plant of a second transgene encoding Barstar which is expressed at least in all those cells wherein the first transgene is expressed.

In the Barnase/Barstar system for altering and restoring plant phenotype the first transgene, the Barnase gene is believed to interfere with a large number of endogenous gene products in a non-specific way, rather than by interaction with a preselected endogenous gene product. The restoration of male-fertility is based on a direct interaction of Barstar with Barnase. In general terms, fertility restoration according to this system is based on direct interaction of the restoration gene product with the sterility gene product in the plant cell. This is one of the best described phenotype restoration systems known in the art. However, a drawback of the Barnase/Barstar system is that its application is limited to phenotypes which allow disruption of cell structures by cell death. Phenotypes that require more subtle modification of plant cell functioning, such as alteration of flower colour, fruit ripening, and the like, are outside the scope of this system.

Many systems for altering plant phenotypes are based on inhibition of endogenous plant genes. Examples thereof include but are not limited to disease-resistance, flower colour, fruit-ripening, male-sterility, and the like. It is an object of the invention to provide a phenotype restoration or moderation system that can be used when plant phenotypes have

b en altered due to the expression of a transgene capable of inhibiting expression of a particular endogenous gene.

SUMMARY OF THE INVENTION

The present invention provides a process for the restoration of a plant phenotype that is altered due to a first transgene which when expressed inhibits expression of an endogenous plant gene, by introducing into said plant, or progeny thereof, a second transgene which when expressed is capable of neutralising or partially neutralizing the eff ct caused by the first transgene, whereby said second transgene is expressed at least in those cells involved in the altered phenotype. Preferred in a process according to the invention is a second transgene which encodes a protein or polypeptide gene product that is capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 90%, preferably less than 80%, yet more preferably said second transgene encodes a protein or polypeptide gene product that is not identical in amino acid sequence to the endogenous gene product and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 75%. According to a special preferred embodiment said second transgene is obtainable from a different plant species.

The invention further provides a process for the restoration of fertility in a plant that is male-sterile due to a first transgene which when expressed inhibits expression of an endogenous plant gene required for pollen development or functioning, by introducing into said plant a second transgene capable of neutralising the effect caused by the first transgene, whereby said second transgene is expressed in all cells in which the first transgene is expressed. Preferred in a process according to the invention said second transgene encodes a protein or polypeptide gene product that is capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene and wherein the nucleotide sequence identity of the transcripts encoded by

the second transgene and the first transgene is less than 90% , preferably less than 80%, more preferably said sec nd transgene encodes a protein or polypeptide gene product that is not identical in its amino acid sequence to the endogenous gene product and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 75%.

According to a special preferred embodiment said second transgene is obtainable from a different plant species.

According to a special embodiment the process according to the invention said first transgene is an antisense gene which when expressed inhibits expression of an endogenous flavonoid biosynthesis gene and said second transgene encodes a flavonoid biosynthesis enzyme capable of substituting the function of the corresponding flavonoid biosynthesis enzyme encoded by the said endogenous gene. Preferred according to this embodiment is a first transgene which is an antisense gene inhibiting expression of an endogenous chalcone synthase gene and said second transgene encodes a chalcone synthase capable of substituting the function of the chalcone synthase encoded by the said endogenous gene. Especially preferred first transgenes and second transgenes for the restoration or moderation of male-fertility are those obtainable from table 1 in this specification.

Preferred in a process according to the invention is the process wherein said second transgene is introduced into the progeny of said plant by cross-pollination of a parent of said plant with pollen comprising said second transgene.

The invention further provides a process for obtaining fertile hybrid seed of a self-fertilizing plant species, comprising the steps of cross-pollinating a plant S which is male-sterile due to a transgene which when expressed inhibits expression of an endogenous gene required for normal pollen development or functioning, with a plant R which is male-fertile and comprises a transgene that encodes a protein r polypeptide product capable of substituting the function of th protein or polypeptide product encoded by the said ndogenous g ne. Preferred according to this process is a first transgene which is an antisense chalcone synthase g ne,

the endogenous gene is a chalcone synthase gene, and the second transgene encodes chalcon synthas , wherein the nucleic acid sequence identity of the transcripts encod d by the second transgene and the first transgene is less than 90%, preferably less than 80%, more preferably less than 75%.

The invention also comprises fertile hybrid seed obtained by a process according to the invention, as well as plants obtained from fertile hybrid seed, as well as parts of the plants, such as a bulb, flower, fruit, leaf, pollen, root or root culture, seed, stalk, tuber or microtuber, and the like.

The invention further comprises plants, as well as parts thereof, which harbour a chimeric gene which when expressed produces a protein or polypeptide product capable of substituting the function of a polypeptide or protein encoded by an endogenous gene of said plant, wherein the nucleotide sequence identity of the transcripts encoded by the transg ne and the endogenous gene is less than 90%, preferably less than 80%, more preferably less than 75%.

DESCRIPTION OF THE FIGURES

Figure 1. A representation of plasmid MIP289 harbouring an expression cassette with multiple cloning site, which can be suitably used to insert foreign genes and antisense genes for expression in anthers of plant cells; CHI PB: chalcone isomerase B promoter; NOS tail: transcription termination signal derived from the nopaline synthase gene of Agrobacterium.

Figure 2. Same plasmid as in figure 1, wherein the expression cassette contains a hybrid promoter based on the 35S promoter of cauliflower mosaic virus, and a so-called anther box (for details of promoter, vide Van der Meer, et al, 1992, sub)

Figure 3. Crossing scheme for obtaining fully male-fertile hybrid seed according to the invention; plant S (Ssrr): maternal male-sterile line heterozygous for the sterility gene which when expressed inhibits expression of an endogenous plant gene required for pollen development or functioning; plant R: pollinator line heterozygous for a

restoration transgene capable of neutralising the effect caused by the first transgen .

Figure 4. Similar crossing as in Figure 3, except f r the pollinator line which is homozygous for the restoration gene.

Figure 5. Binary vector pFBP125. This is a pBIN19 based vector with an insert comprising a chs gene from Arabidopsis thaliana between a hybrid promoter fragment comprising the CaMV 35S RNA promoter in which an anther-box (AB) has be n inserted, and the nos-termination region of Agrobacterium tumefaciens.

Figure 6. Binary vector pFBP130. This is a pBIN19 based vector with an insert comprising an chs gene from Arabidopsis thaliana between a promoter fragment of the chs-A gene of Petunia hybrida and the nos-termination region of Agrobacterium tumefaciens.

Figure 7. Southern analysis of plant DNA of several petunia lines containing: (a) petunia anti-sense chs construct (T29), (b) Arabidopsis sense chs gene construct (-T36004), (c) both constructs (a) and (b) (T38002 and T38007) and wild-type (W115) probed with ^{32}P-labelled Arabidopsis chs DNA (o/n exposure -80 degr. Celsius). The Arabidopsis chs genes are clearly visible in T38002 (several strong bands), T38007 (several strong bands) and T36004 (one strong upper band), whereas there is only slight cross-hybridization with the endogenous petunia chs genes or antisense petunia chs genes (faint bands in the lanes of T38002, T38007, T29 and W115 and the antisense gene in T29).

Figure 8. Northern analysis of messenger RNA of the same plants as in Fig. 7, including now T38005. Probed with petunia chs DNA; 6 days exposure -80 degr. Celsius). The chs mRNA are clearly visible in the lanes of T36004 and W115 as

expected. In none of the antisense plant lines (T29, T38002, T38005, T38007) could a petunia mRNA be detected, as could have been expected as well.

Figure 9. Northern analysis as in Figure 8, except that the blot was probed with Arabidopsis chs DNA, o/n exposure at -80 degrees Celsius. At o/n exposure the Arabidopsis chs MRNA is only detected in the lane of T36004. However, upon gross overexposure some very faint bands could be detected in the lanes of the double transgenic lines T38002, T38005 and T38007.

DETAILED DESCRIPTION

The instant invention will be illustrated by outlining in more detail the findings that are obtained when performing experiments aimed at restoration of male-fertility in plants that were made male-sterile by the expression in the tapetal cell layers of a chalcone synthase transgene which was placed in the reverse orientation with respect to the promoter. The details of the gene constructs and the male-sterile plants obtained therewith are described in Van der Meer et al., (1992, The Plant Cell 4, 253-262).

It was shown that expression of an antisense CHS gene in the anthers of transgenic plants caused inhibition of normal pollen functioning as a result of which the plant were unable to self-pollinate. The transgenic male-sterile plants w re found to be entirely female-fertile and could be made to set seed by cross-pollination with a male-fertile pollinator line. It was concluded that the antisense chs plants can be suitably used for the production of hybrid crops.

In the experiments that underlie the present invention a male-sterile Petunia hybrida plant S which is transgenic for an antisense CHS gene from Petunia hybrida under the control of regulatory sequences that provide for expression of the transgene in anthers of the plants, is cross-fertilised with a Petunia hybrida plant R that contains a transgene obtainable from the chs gene of Arabidopsis thaliana which is under the control of regulatory sequences that provide for expression of the transgene in anthers of the plants.

Of the pollinator plants R, harbouring nly the transgen from _Arabidopsis thaliana_ the majority is not male-sterile as might have been expected from the finding that transgenes can inhibit the expression of resident genes encoding homologous gene products. This so-called co-suppressive effect has been established for a number of genes including a _chs_ transgene obtainable from _Petunia hybrida_ and re-introduced into petunia plants (Napoli C. _et al_., 1990, The Plant Cell _2_, 279-289; Van der Meer I. _et al_., 1992, Plant Cell _4_, 253-262). It has also been disclosed that expression of a _chs_ transgene placed in the sense direction under the control of its promoter gives rise to male-sterile plants, just as expression of an antisense _chs_ gene does, provided expression of the transgenes occurs at least in the tapetal cell lay r of the anthers of the plants (PCT/NL92/00075, which is herewith incorporated by reference in this specification, with the proviso that the definitions in that application do not apply to the description of this invention and the claims attached thereto at present or after amendment).

The finding that the introduction of a divergent _chs_ gene, such as the one from _Arabidopsis_, does not markedly inhibit the production of chalcone synthase in the transgenic plants indicates, that significant co-suppressive effects are absent if a transgene is selected that encodes a transcript that is sufficiently divergent from the endogenous gene transcript.

The crossing of male-sterile plant S, which is heterozygous for the sterility gene (Ssrr) with plant R, homozygous for the restoration gene (ssRR) yields hybrid seed SR of which 50% contains in addition to the endogenous _chs_ gene and the _Arabidopsis chs_ gene in the sense orientation, the antisense _chs_ gene from _Petunia hybrida_. Contrary to expectation, it will be found, that a percentage of the progeny plants grown from the hybrid se d (50% SsRr; 50% ssRr) harbouring both the transgenes is again capable of self-fertilization in spite of the fact that about 50% also inherited the sterility gene.

To establish the nature of the restored phenotyp a transcript specific primer extension experiment is carried

out on CDNA obtained from young anthers. Attempts to visualize radioactive extension products corresponding to the first (petunia chs) transgene transcript fails, which can be expected in view of the restored phenotype. Applying equal radio-illumination times it is also impossible to detect the presence of the endogenous chs gene transcript, whereas an extension product of about 1.4 kb obtained with the primers represented as SEQIDNO: 1 and SEQIDNO: 2 corresponding to Arabidopsis chs transgene transcript can be clearly detected under these conditions. The corollary of these experiments is that the endogenous gene transcript and the almost identical petunia transgene transcript interact, presumably by basepairing, as a consequence whereof these transcripts are not expressed and probably degraded in the plant nucleus. It is presumably due to the nucleic acid sequence divergence of the Arabidopsis transgene with respect to both the endogenous petunia gene, as well as the petunia transgene, that the former does not interact with any of the transcripts encoded by the latter two genes. The nucleic acid sequences of the Arabidopsis transgene and the Petunia gene transcripts diff r at least 30% in the protein encoding region, presumably even more if the non-translated regions of the transcript are taken into account. Hence, the nucleic acid divergence of the transcript is deemed responsible for its translatability in the plant cell, thereby producing a fully active chalcone synthase which substitutes the endogenous chalcone synthase. As a result male-fertility is restored in a percentage of the progeny plants despite the fact that about 50% thereof contain the sterility transgene.

Apparently, the high degree of nucleic acid sequence identity of the first (petunia) chs transgene antisense transcript and the endogenous (petunia) chs transcript favours the interaction of these molecules, probably causing them to be degraded, while the second chs transgene transcript from Arabidopsis thaliana which is at the most 75% identical on the nucleic acid level (see Table 1), is produced in sufficient quantities to be translated into a fully functional (heterologous) chalcone synthase capable of restoring the plant's altered phenotype. We therefore main-

tain that the restoration of the male-fertility phenotype is due to complementation on the enzyme level.

This is believed to be the first observation of partial phenotype restoration, or phenotype moderation, in plants, wherein the production of an endogenous protein product is blocked and wherein the function of that protein product is substituted by a protein product similar (not necessarily identical) on the amino acid level, but encoded by a nucleotide sequence which is different on the nucleic acid level. This finding may have interesting applications in the genetic modification, restoration, or moderation of plant phenotypes, in and outside the area of hybrid seed production. For example, it is now feasible to silence endogenous enzymes, and substitute such enzymes by enzymes with different properties, such as a different substrate specificity, mode of regulation, and the like. Such substitutions may bring about subtle, yet interesting, changes in the biochemical pathway in which the endogenous enzyme is involved.

The various aspects of the invention are outlined in more detail below.

The invention can be worked with any phenotype alteration system that involves an inhibitory gene of the antisense type, such as described in EP 240 208 A2, directed against an endogenous gene. Evenly so, it can be worked with an inhibitory gene of the sense type, which work by the as yet not fully understood mechanism referred to as co-suppression, disclosed in Napoli _et al_., 1990, _supra_. Examples of such phenotypes include, but are not limited to disease-resistance, drought-resistance, flower colour, fruit ripening, and the like.

The restoration gene must encode a transcript that is sufficiently divergent from both the endogenous gene transcript as well as the inhibitory transgene transcript and y t encodes a protein or polypeptide capable of substituting the function of the endogenous gen product. Phenotype restoration can be made absolute. Alternatively, phenotype restoration may be made not absolute; in this case it is preferr d to speak of partial phenotype restoration or 'phenotype moderation'. If absolute phenotype restoration is aimed at,

the divergence of the transcript must diverge preferably by more than 20%, that is the nucleic acid identity of the restoration transcript with either the inhibitory transgene transcript or the endogenous gene transcript does not exceed 80%, preferably it does not exceed 75%. Depending on the level of moderation desired, optimal moderation can be achieved by making transgenes with different levels of divergence and selecting the desired phenotype. In case phenotype restoration is not required to be absolute, or desired to be not absolute, divergence of the restoration transgene transcript should not exceed 20%, preferably it should not exceed 10%. The latter is referred to as phenotype moderation.

Likewise, phenotype alteration systems that involve inhibitory genes of the ribozyme type directed as sequence specific endo-ribonucleases against an endogenous gene transcript, as disclosed in US Patent 4,987,071, may be restored with a transgene according to the invention, with the proviso that the restoration gene encodes a transcript that is lacking the recognition and/or cleavage consensus of the ribozyme. Phenotype moderation should be possible using this kind of inhibitory transgenes as well, although manipulating the recognition and cleavage sequence of the restoration gene to affect its affinity for the ribozyme may require some trial and error.

The choice of the restoration gene

As a rule the restoration gene must not give rise to a transcript that is identical to the endogenous gene transcript. Preferably, the restoration gene transcribed region is as much divergent from the transcribed region of the endogenous gene as possible, while the protein product encoded by said transcript is identical, or almost identical. It is well known in the art that each amino acid can b encoded by a more than one codon; this fact, referred to as the degeneracy of the genetic code, stems from the fact that there are about 20 different amino acids, which are encoded by triplets of four different bases, yielding a total of 64 possible codons. Three codons comprise stop signals for translation, so that

in actual fact 61 codon specify about 20 amino acids. Roughly spoken, every third base may be changed in a coding region without affecting the amino acid sequence of the protein. This means that the transcribed region of a restoration gene can at least diverge 33% from the endogenous gene. But, sinc a gene transcript generally comprises non-translated regions flanking the coding region on both sides, even further nucleic acid divergence may be achieved in order to avoid interaction of the restoration gene transcript with the endogenous gene transcript or the first transgene transcript.

Furthermore, still greater divergence may be achieved if one takes into account the fact that two proteins may differ in their amino acid sequence, while retaining their physiological activity in the plant cell. Although it is not established to what extent this may be, it may be assumed that proteins which have conservative amino acid replacements in 10% of their amino acids, will still be capable of performing their physiological role. Altogether, it will be clear to someone skilled in the art that a restoration gene according to the present invention need not be more identical to its endogenous counterpart than about 40-50% on the nucleic acid level.

Some aspects of the invention will be further illustrated with male-sterility as exemplifying phenotype.

<u>Obtention of a male-sterile maternal line S</u>

Any male-sterile plant phenotype that is due to expression of an inhibitory gene of one of the types mentioned in the preceding paragraphs can be restored by a restoration gene according to the invention.

Typical examples of how genes can be identified that are essential for pollen development or pollen functioning is given <u>inter alia</u> in WO89/10396 and WO90/08828. Once such genes are isolat d they can be expressed or overexpressed in th sense or antisense orientation in those cells required for pollen development or functioning. In order to achieve expression in those cells that are necessary for pollen development, genes are placed under the control of promoters that are expressed in stamen cells (including filaments and

anth rs), or more specifically in anthers, or even more specifically in tapetal cell layers thereof. A distinction should be made to sterility genes that are disruptive to general plant cell functioning or viability on the one hand, and genes that disrupt plant metabolism to the extent that it disrupt pollen development or functioning without drastically affecting plant viability on the other hand. The antisense chalcone synthase gene is one of the latter category; consequently, it is not necessary for the latter type sterility gene to be expressed exclusively in stamen cells through the use of stamen-specific promoters. Sterility genes of the former type, i.e. the general plant cell disrupters, must not be effective inside plant structures essential for survival of the plant. Methods for isolating promoters that provide for proper expression patterns of these genes are also described in both WO89/10396 and WO90/08828, which are herewith deemed incorporated by reference.

For reasons of illustration the maternal male-sterile line is represented as being heterozygous for the sterility gene. However, it will be clear that fully fertile hybrid seed can be obtained also if the maternal line is homozygous for the sterility gene. International Patent Application PCT/NL92/00075, discloses a method for obtaining homozygous male-sterile plants, by selfing male-sterile plants harbouring one copy of an antisense chs gene, whereby the pollen that are arrested in their development are made to germinate on pistils in the presence of flavonoids. The seed obtained from this selfing can be grown into homozygous male-sterile maternal plant lines, which can optionally be propagated *in vitro* first, and then used as such in hybrid seed production by cross-pollination with a pollinator line, which may be heterozygous or homozygous for the restoration gene according to the invention.

Plant transformation

Introduction of sterility genes, herbicide resistance genes or restoration genes into plants, is achieved by a any one of the following techniques, the choice of which is not critical to the present invention.

Generally, us ful methods ar th calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1982, Nature 296, 72-74; Negrutiu I. et al, June 1987, Plant Mol. Biol. 8, 363-373), electroporation of protoplasts (Shillito R.D. et al., 1985 Bio/Technol. 3, 1099-1102), microinjection into plant material (Crossway A. et al., 1986, Mol. Gen. Genet. 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. et al., 1987, Nature 327, 70), infection with viruses and the like.

Preferred according to the invention is the use of Agrobacterium-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP-A 120 516 and U.S. Patent 4,940,838).

Subsequently, receptive plant cells or are selected for the presence of one or more markers which are encoded by plant expressible genes co-transferred with the plant expressible gene according to the invention, whereafter the transformed material is regenerated into a whole plant. Alternatively, pollen cells are transformed, for instance by coated-particle acceleration, and used to pollinate receptive plants.

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells. Presently, preferred methods for transformation of monocots are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al, 1989, Nature 338, 274-276). Transgenic maize plants have been obtained by introducing the Streptomyces hygroscopicus bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm et al, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by sel cting only the aged compact and nodular

embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil I., et al, 1990, Bio/Technol. 8, 429-434). Herbicide resistant fertile wheat plants were obtained by microprojectile bombardment of regenerable embryogenic callus (Vasil V. et al, 1992, Bio/technol. 10, 667-674).The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially imp rtant crops such as corn are also amenable to DNA transfer by Agrobacterium strains (Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434).

Marker genes

Suitable marker genes that can be used to select or screen for transformed cells, can be selected from any one f the following non-limitative list: neomycin phosphotransphe-rase genes conferring resistance to kanamycin (EP-B 131 623), the hygromycin resistance gene (EP 186 425 A2) the Glutathione-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine synthetase conferring upon overexpression resistan-ce to glutamine synthetase inhibitors such as phosphinothri-cin (WO87/05327), the acetyl transferase gene from Streptomy-ces viridochromogenes conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) conferring tole-rance to N-phosphonomethylglycine, the bar gene conferring resistance against Bialaphos (e.g. WO91/02071), and the like. The actual choice of the marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice.

The marker gene and the gene of interest do not n cessa-rily have to be linked, since co-transformation of unlinked genes (U.S. Pat nt 4,399,216) is also an efficient process in plant transformation.

Gene expression

The expression pattern required for the restoration gene depends on the expression pattern of the inhibitory transgene. The latter in its turn is dependent on the phenotype alteration aimed at. Thus, for modifying the fruit ripening phenotype in a plant, an inhibitory gene bringing about said alteration must at least be expressed in the fruits of said plant. Restoration or moderation can be achieved by an expression pattern that comprises at least the expression pattern of the inhibitory transgene.

Multiple transgenic plants

To obtain transgenic plants harbouring more than one gene a number of alternatives are available, the actual choice of which is not material to the present invention:

A. the use of one recombinant polynucleotide, e.g a plasmid, with a number of modified genes physically coupled to one selection marker gene.

B. Cross-pollination of transgenic plants which are already capable of expressing one or more chimeric genes coupled t a gene encoding a selection marker, with pollen from a transgenic plant which contains one or more gene constructions coupled to another selection marker. Afterwards the seed, which is obtained by this crossing, is selected on the basis of the presence of the two markers. The plants obtained from the selected seeds can afterwards be used for further crossing.

C. The use of a number of various recombinant polynucleotides, e.g. plasmids, each having one or more chimeric genes and one other selection marker. If the frequency of cotransformation is high, then selection on the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.

D. Consecutive transformations of transgenic plants with new, additional genes and selection marker gen s.

E. Combinations of the above mentioned strategies.

The actual strategy is not critical with r sp ct to the described invention.

selection of hybrid seed

It is known in the art that, the need to separat hybrid seed from non-hybrid seed can be avoided if the self-pollinators can be destroyed, for example by using an antibiotic, preferably a herbicide. This requires that the maternal mal - sterile line is resistant to this antibiotic or herbicide due to the presence of transgene coding therefor.

The herbicide resistance gene may be introduced into th maternal line simultaneously with the sterility gene according to the invention by genetic transformation with a multigene construct. However, the herbicide resistance gene may be introduced into the maternal line after the introduction of the sterility gene.

It may be advantageous to introduce the herbicide resistance trait into the plant intended to use as maternal parent line prior to the introduction of the sterility gene. This simplifies the creation of plants that are homozygous for the herbicide resistance phenotype which may be advantageous. Then, plants provided subsequently with the sterility gene, may be cross-pollinated with a pollinator plant containing a restoration gene according to the invention. Suitable herbicides can be selected from any one listed under the heading marker genes.

Advantages and industrial application

The process according to the invention is particularly useful for the production of hybrid progeny that is fully male-fertile.

In a conventional process of producing hybrids from self-fertilising crops a transgenic (heterozygous) nuclear male-sterile plant line S (Ssrr) may be crossed with a male-fertile plant line R (ssrr) to yield hybrids that are 50% fertile (ssrr) and 50% sterile (Ssrr). Consequently, if such hybrid crops were grown in the field directly, 50% of the acreage would consist of plants that must be cross-fertilised in order to set seed, which may have significant yield reducing eff cts for those crops that rely on the s tting of fruit or seed for their commercial value. Examples of such crops include but are not limited to cereals and oil seed rape.

Thus, the present invention is specially suitable for the hybridization of naturally self-fertilizing crops by crossing a maternal line which is male-sterile due to the expression of a first transgene capable of inhibiting expression of an endogenous plant gene essential to normal pollen functioning, and a pollinator line containing a second transgene capable of neutralising the effect caused by the first transgene. Although 50% of the hybrid progeny is heterozygous for the sterility gene, the presence of the restoration or moderation gene ensures fertility of the progeny that is closer to that of the wild type lines.

The specific advantages of this hybridization system reside in the fact that it can be used in combination with any sterility system that makes use of transgenes inhibitory to endogenous genes. As a consequence the phenotype can be determined predominantly by the nature of the gene product, rather than the specificity of the expression pattern.

All references cited in this specification are indicative of the level of skill in the art to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein incorporated by reference as if each of them was individually incorporated by reference.

The Examples given below are just given for purp - ses of illustration and do not intend in any way to limit the scope of the invention.

EXAMPLE 1

Construction of a chiPB/as-chs and a chalcone isomerase B promoter chs gene construct (chiPB/chs-At)

The chiPB/as-chs construct comprises a chs cDNA fragment from *Petunia hybrida* fused in the antisense orientation to a chalcone isomerase B promoter fragment. The chiPB/chs-At construct comprises a chs cDNA fragm nt from *Arabidopsis thaliana* fused in the sense orientation to a chalcone isomerase B promoter fragment.

A 1.7 kb promoter fragment from the anther-specific $chiP_B$ promoter (Tunen, A.J. Van., Mur, L.A., Brouns, G.A., Rienstra, J.D., Koes, R.E. and Mol. J.N.M., 1990, The Plant

Cell 2, 393-401) and a 0.2 kb NOS tail isolat d from plasmid pBI101.1 (Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). EMBO J. 6, 3901-3907) are cloned into the plasmid pUC19 (Messing, J., 1978, Recombinant DNA Technical Bulletin NIH Publication No. 79-99, 2, 43-48) yielding the recombinant plasmid MIP289 (Figure 1).

A 1.4 kb BamHI chs fragment is isolated from plasmid pTS21 (Van der Meer et al., 1992, supra) and cloned into plasmid MIP289 digested with BamHI. A clone with the chs fragment in an antisense orientation is selected on the basis of the asymmetric SstI restriction enzyme site. Subsequently, this fragment is subcloned as a HindIII/EcoRI fragment into the binary vector Bin19 (Bevan, M. (1984) Nucl. Acid Res. 12, 8711-8712) yielding plasmid pAS8.

To isolate a full size Arabidopsis chs cDNA, single stranded cDNA is synthesized on 10 µg RNA isolated from young Arabidopsis thaliana ecotype Landsberg erecta flower buds, by priming with an 17-mer oligo-dT primer (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbour, NY: Cold Spring Harbour Laboratory). A set of two additional primers based n (Feinbaum, R.L., and Ausubel, F.M. (1988). Mol. Cel. Biol. 8, 1985-1992) with the sequence based on the 5' region (prim r I = SEQIDNO: 1; GCGGATCCGTATACTATAATGGTGATGG) and 3' region (primer II = SEQIDNO: 2; GAGGATCCTTAGAGAGGAACGCTGTGCAAGAC) of the Arabidopsis chs gene are used for the initial polymerase chain reaction (PCR) analysis. The PCR reaction is performed in 100 µl PCR buffer (10 mM Tris, pH 8.3, 50mM KCl, 2.5 mM $MgCl_2$) containing 50 pmole primers, and 200 µM of each deoxynucleotide triphosphate. Amplification involved 30 cycles of a standard cycle for homologous primers. Amplified CDNA is fractionated on a 1% agarose gel and a 1.4 kb band is isolated and subcloned as a BamHI fragment (sites present in the 5' and 3' primers) in pAS8 after digestion with BamHI to remove the petunia chs CDNA. The orientation and proper cloning of the Arabidopsis chs CDNA into PAS8/BamHI is checked by a detailed restriction enzyme analysis and sequence analysis; the correct plasmid is called pAS9.

Example 2

Transformation of tobacco plants

The plasmids pAS8 and pAS9 are transferred from E. c li JM83 (Messing et al, 1978, supra) to Agrobacterium tumefaciens strain LBA 4404 (Hoekema A. et al., 1983, Nature 303: 179-180) by triparental mating (Rogers, S.G., and Fraley, R.T., 1985, Science 227, 1229-1231), using a strain containing plasmid pRK2013 (Ditta et al., 1980, Proc. Nat. Ac. Sci. USA, 12, 7347-7351). Transformed tobacco plants are obtained by the standard leaf-disc transformation method (Horsch et al., 1985, Science 227, 1229-1231). After cultivation with the A. tumefaciens strains harbouring either pAS8 or pAS9, the tobacco leaf discs are grown on MS plates containing 3 μg/ml kinetin, 500 μg carbenicillin and 200 μg kanamycin. Plants obtained are checked for transformation on the basis of resistance for kanamycin and by Southern blot analysis using an npt fragment as a probe. After shoot and root induction plants are put on soil and transferred to the greenhouse. Plants are grown under in the greenhouse at 21°C at a 16 hours light, 8 hours dark regime.

Example 3

Analysis of transgenic plants expressing the antisense chs construct

Transgenic tobacco plants containing the chimeric pAS8 gene construct (Petunia antisense chs) are investigated for fertility by self-pollination. At least one plant is almost completely sterile and shows a seed set of less than 1% in selfings. Furthermore the pollen grains of this plant are morphologically aberrant, as was also published by Van der Meer et al. (1991) and are not able to germinate in an in vitro germination assay. This plant is designated S1 and contains only one copy of construct pAS8 in its genome.

Example 4

Analysis of transgenic plants expressing the chimeric Arabidopsis chs construct

From a number of 15 transgenic tobacco plants containing plasmid pAS9, one plant expressing the Arabidopsis chs cDNA

in young anthers is selected by RNAse protection experiments using RNA isolated from young anthers. This plant is designated R1.

Example 5

Crossing of S1 and R1 restores fertility

A cross is made between S1 (genotype Ssrr) and R1 (genotype ssRr) and the offspring of this cross is grown to mature plants. Based on their genotype four classes of plants can be distinguished: Ssrr, SsRr, ssRr, and ssrr (see also Figure 2). It can be observed that plants containing the restoration gene, i.e. the *Arabidopsis chs* gene (SsRr) are able to set seed after self-pollination despite the presence of a sterility gene (Ss). Light-microscopical analysis sh ws that these plants have pollen that are morphologically normal whereas Ssrr plants have aberrant pollen. All plants containing both the sterility gene construct pAS8 and the restoration gene construct pAS9 show restoration of fertility as can be demonstrated by self-pollination experiments. In a control cross between S1 and an untransformed tobacco plant only 50% of the offspring is able to set seed after self-pollination as can be expected on the basis of the fact that S1 has a copy of construct pAS8 integrated in its genome.

EXAMPLE 6

The following table provides data about chalcone synthase genes from various plant species and the nucleic acid identity of the amino acid coding regions: reference sequence is *Petunia hybrida* V30 chalcone synthase gene. Best match is given at a minimum sequence of 1000 bp.

TABLE 1

Comparison of NA sequence identity of *chs* genes

source	gene designation	identity (%)
P. hybrida V30	*chs*	100
P. hybrida	*chs*A	98
P. hybrida	*chs*J	82
P. hybrida	*chs*H	79
P. hybrida	*chs*D	77
P. hybrida	*chs*F	78
P. hybrida	*chs*G	76

L. esculentum	TCHS1	83
L. esculentum	TCHS2	83
P. sativum	PSCHS1	76
P. sativum	PSCHS2	74
P. sativum	PSCHS3	73
Soybean	CHS gene 3	74
G. max	CHS gene 2	73
Parsley	CHS1	72
M. incana	CHSy	71
A. thaliana	**Atchs**	71
Mustard	SasCHS3	72
Mustard	SasCHSsg	70
Mustard	SasCHS1	69
Antirrhinum majus	AmCHS	74
Pinus sylvestris	PsCHSs	70
Hordeum vulgare	X58339	68
Zea mays	Zmc2cs	67
Zea mays	Zmwpcs	67

Boldface: gene fragments that are used as sterility and restoration gene respectively, in this disclosure.

Other suitable combinations of sterility genes and restoration genes can be selected from this table.

EXAMPLE 7

Partial fertility restoration in male-sterile plants by re-transforming male-sterile plants with a divergent restoration gene construct

Petunia W115 plants were transformed with a sterility gene construct comprising the promoter region of the petunia chs gene linked to the coding region of the petunia chs gene in antisense orientation. This gene construct, designated VIP176 (Krol A.R. van der et al., 1990, Plant Molecular Biology 14, 457-466) was used to transform the petunia line W115 and a self-sterile plant was selected and designated T17002. This self-sterile flavonoid depleted plant, T17002, was cross-pollinated with a W115 plant and among the progeny a plant was selected, which was kanamycin sensitive but still self-sterile and depleted for flavonoids; this plant was designated T29. This sterile, kanamycin sensitive plant, T29, was used for a second transformation with pFBP125 ($P_{CaMV35SAB}$/-CHS_{At}, yielding the 39000 plants, not discussed further) or pFBP130 ($P_{CHS,pet}$/CHS_{At}, rendering the 38000 plants, see below).

This approach was successful as 7 transgenic 38000 plants were obtained which contain both the sterility gene

construct (chs-antisense from petunia) as well as the restoration construct (sense-chs from Arabidopsis). Of these plants 5 had flavonol production in the corolla; 2 out these 5 plants were male-fertile (inter alia T38005).

In order to obtain data about the functionality of the Arabidopsis CHS-enzyme in petunia plants, W115 plants wer transformed with Agrobacterium strains harbouring pFBP125 (yielding the 36000 plants, see below). Of 15 transformed plants, 4 plants over-produced flavonols as compared to wild-type (W115) (inter alia T36004, see Table 2).

Plant lines were tested for the presence of the constructs by Southern analysis. Expression of the genes was verified by Northern analysis.

Table 2 summarizes the results for 6 petunia lines: from top to bottom are given Southern data, obtained by probing with petunia chs probes and Arabidopsis chs probes; North rn data, obtained by probing with both aforementioned probes, corolla pigmentation (flavonol staining); and fertility determination. The genetic backgrounds of the petunia lin s are as follows: W115 - wild-type petunia plants (non-transgenic); T29 - $P_{CaMV35SAB}$-antisense petunia chs (transgenic for sterility gene); T38002, T38005, T38002 - P_{chs}-antisens petunia chs + P_{chs}-A.thaliana chs (transgenic for sterility gene and restoration gene); T36004 - $P_{CaMV35SAB}$-A. thaliana chs (transgenic for the restoration gene only).

As indicated in the table lines W115, which is 100% fertile, and T29, which is entirely unable to self-pollinate, performed as expected (see PCT/NL92/00075). The double-transgenic lines T38002, T38005 and T38007, which contain both the sterility gene and the fertility gene, had only a partially restored fertility; for T38005 seed-set was about 10-20% of the wild-type W115. These data correspond well with the presence of only slight amounts of flavonols (see below). Moreover, the presence of flavonols was depend nt on th presenc of the Arabidopsis chs gene, as was confirmed by Southern data using the Arabidopsis chs PCR fragment as a probe. The Arabidopsis chs probe was only weakly capable of cross-hybridizing with the petunia chs gene and vice versa (Fig. 7).

Th Northern data on mRNA of corolla's corresponded with the Southern data, except that the Arabidopsis chs-messenger RNA of plant lines T38002, T38005 and T38007, when probed with the Arabidopsis chs-probe, could only be detected after gross over-exposure; this is probably due to weak expression of the Pchs-Arabidopsis chs gene construct in corolla's. The Northern data for lines T38002, T38005 and T38007 seem in accordance with production of low amounts of flavonoles in these lines, which, in turn, might explain the fact that the sterility was restored only partially (only 10-20% seed set for T38005 as compared to W115). In order to restore fertility it is necessary that the restoration gene construct (such as in pFBP125 and pFBP130) is expressed in either the male reproductive organs or the female reproductive organs or in both. Although expression of the restoration gene in corolla's provides an initial indication of fertility restoration it will be necessary to establish expression of the Arabidopsis gene in either of the reproductive organs. We anticipate that the T38005 plant expresses the Arabidopsis gen in either of the reproductive organs, and Northern analysis is in progress to confirm this.

The proper functioning of the Arabidopsis CHS-enzyme was established by comparing flavonol production in W115 with T36004, which contains, in addition to its endogenous chs-gene, copies of the Arabidopsis chs-gene. As is indicated, corolla's of T36004 produced far more flavonols (+++ = dark orange, after staining for flavonols) than W115 corolla's (+ = pale orange). As expected, male-sterile line T29 did not produce detectable amounts of flavonols (- = purely white corolla's), whereas the corolla's of T38002, T38005, which was partially fertility-restored, and T38007 produced slight amounts of flavonols in the corolla (+/- = beige or very pal orange).

The high flavonol levels observed in corolla's of T36004 corr spond w ll with the Northern data obtained for that plant line, indicating abundant levels of the Arabidopsis chs-messenger RNA in these lines (see Fig. 9). It is, therefore, clear that the Arabidopsis CHS-enzyme is fully functional in petunia plants and, in principle, capable of

substituting the function of endogenous CHS.

Deposited microorganisms

On October 14, 1993, two E. coli JM101 strains, one harbouring pFBP125, and one harbouring pFBP130 have been deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, under accession number CBS 543.93 and CBS 544.93, respectively.

	W115	T29	T38007 T38005 T38002	T36004
outhern blotting CHS petunia probe	+ endogenous CHS	+ endogenous CHS + antisense CHS	+ endogenous CHS + antisense CHS	+ endogenous CHS
CHS At probe[1]	-	-	+	+
orthern blotting CHS petunia probe	+	-	-	+
CHS At probe	-	-	+/-[2]	+++
lavonol staining n corolla[5]	+	-	+/-	+++
ertility	100%	-	10% - 20%[3]	100%[4]

e also Figure 7.
signal was detected only after gross over-exposure; see also Figure 8 and 9.
'elfed seed is used for linkage analysis in an out-crossing in order to establish agreement f
: pr sence of the petunia antisense gene (self-sterile), the sense Arabidopsis chs gene (male-
:tile, both the petunia antisense chs and the Arabidopsis chs gene (partially male-fertile), and
transgenes (fertile).
36004 is crossed with homozygous T29 to perform a similar kind of linkage analysis.
'lavonol staining is specific for quercetin and dihydro-kaempferol (aglykones) and is performed
:ording to Sheahan J.J. and Rechnitz G.A., 1992, BioTechniques 13, No. 6, 880-883.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MOGEN International N.V.
(B) STREET: Einsteinweg 97
(C) CITY: LEIDEN
(D) STATE: Zuid-Holland
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): NL-2333 CB
(G) TELEPHONE: (0)31.71.258282
(H) TELEFAX: (0)31.71.221471

(ii) TITLE OF INVENTION: Genetic Restoration of Plant Phenotypes

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana
(B) STRAIN: landsberg erecta
(F) TISSUE TYPE: Flower buds

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Feinbaum, R L
Ausubel, F M
(B) TITLE: Transcriptional regulation of the Arabidopsis thaliana chalcone synthase gene
(C) JOURNAL: Mol. Cell. Biol.
(D) VOLUME: 8
(F) PAGES: 1985-1992
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCGGATCCGT ATACTATAAT GGTGATGG 28

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana
(B) STRAIN: landsberg erecta
(F) TISSUE TYPE: flower buds

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Feinbaum, R L
Ausubel, F M
(B) TITLE: Transcriptional regulation of the Arabidopsis thaliana chalcone synthase
(C) JOURNAL: Mol. Cell. Biol.
(D) VOLUME: 8
(F) PAGES: 1985-1992
(G) DATE: 1988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAGGATCCTT AGAGAGGAAC GCTGTGCAAG AC 32

CLAIMS

1. A process for the restoration of a plant phenotype that is altered due to a first transgene which when expressed inhibits expression of an endogenous plant gene, by introducing into said plant, or progeny thereof, a second transgene which when expressed is capable f neutralising or partially neutralizing the effect caused by the first transgene, whereby said second transgene is expressed at least in those cells involved in the altered phenotype.

2. A process according to claim 1, wherein said second transgene encodes a protein or polypeptide gene product that is capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 90%.

3. A process according to claim 2, wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 80%.

4. A process according to claim 3, wherein the said second transgene encodes a protein or polypeptide gene product that is not identical in amino acid sequence to the endogenous gene product and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 75%.

5. A process according to any one of the claims 1 - 4, wherein said second transgene is obtainable from a different plant species.

6. A process for the restoration of fertility in a plant that is male-sterile due to a first transgene which when expressed inhibits expression of an endogenous plant gene required for pollen development or functioning,
by introducing into said plant a second transgene capable of neutralising the effect caused by the first transgene, whereby said second transgene is expressed in all cells in which the first transgene is expressed.

7. A process according to claim 6, wherein said second transgene

encodes a protein or polypeptide gene product that is capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 90%.

8. A process according to claim 7, wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 80%.

9. A process according to claim 8, wherein the said second transgene encodes a protein or polypeptide gene product that is not identical in its amino acid sequence to the endogenous gene product and wherein the nucleotide sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 75%.

10. A process according to claim 6 - 9, wherein said second transgene is obtainable from a different plant species.

11. A process according to any one of the claims 6 to 10, wherein said first transgene is an antisense gene which when expressed inhibits expression of an endogenous flavonoid biosynthesis gene and said second transgene encodes a flavonoid biosynthesis enzyme capable of substituting the function of the corresponding flavonoid biosynthesis enzyme encoded by the said endogenous gene.

12. A process according to claim 11, wherein said first transgene is an antisense gene inhibiting expression of an endogenous chalcone synthase gene and said second transgene encodes a chalcone synthase capable f substituting the function of the chalcone synthase encoded by the said endogenous gene.

13. A process according to any one of the claims 7 - 12, wherein said first and said second transgene are selected from the group consisting of the chalcone synthase genes obtainable from table 1 in this specification.

14. A process according to any one of the claims 1 to 13, wherein said second transgene is introduced into the progeny of said plant by cross-

pollination of a parent of said plant with pollen comprising said second transgene.

15. A process for obtaining fertile hybrid seed of a self-fertilizing plant species, comprising the steps of cross-pollinating a plant A which is male-sterile due to a transgene which when expressed inhibits expression of an endogenous gene required for normal pollen development or functioning, with a plant B which is male-fertile and comprises a transgene that encodes a protein or polypeptide product capable of substituting the function of the protein or polypeptide product encoded by the said endogenous gene.

16. The process of claim 15, wherein the first transgene is an antisense chalcone synthase gene, the endogenous gene is a chalcone synthase gene, and the second transgene encodes chalcone synthase, wherein the nucleic acid sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 90%.

17. The process of claim 16, wherein the nucleic acid sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 80%.

18. The process of claim 17, wherein the nucleic acid sequence identity of the transcripts encoded by the second transgene and the first transgene is less than 75%.

19. Fertile hybrid seed obtained by the process of claim 15.

20. Plants obtained from seed of claim 19, as well as parts of the plants, such as a bulb, flower, fruit, leaf, pollen, root or root culture, seed, stalk, tuber or microtuber, and the like.

21. A plant, as well as parts thereof, which harbour a chimeric gene which when expressed produces a protein or polypeptide product capable of substituting the function of a polypeptide or protein encoded by an endogenous gene of said plant, wherein the nucleotide sequence identity of the transcripts encoded by the transgene and the endogenous gene is less than 90%.

22. The plant and plant parts of claim 21, wherein the nucleotide sequence identity of the transcripts encoded by the transgene and the endogenous gene is less than 80%.

23. The plant and plant parts of claim 22, wherein the nucleotide sequence identity of the transcripts encoded by the transgene and the endogenous gene is less than 75%.

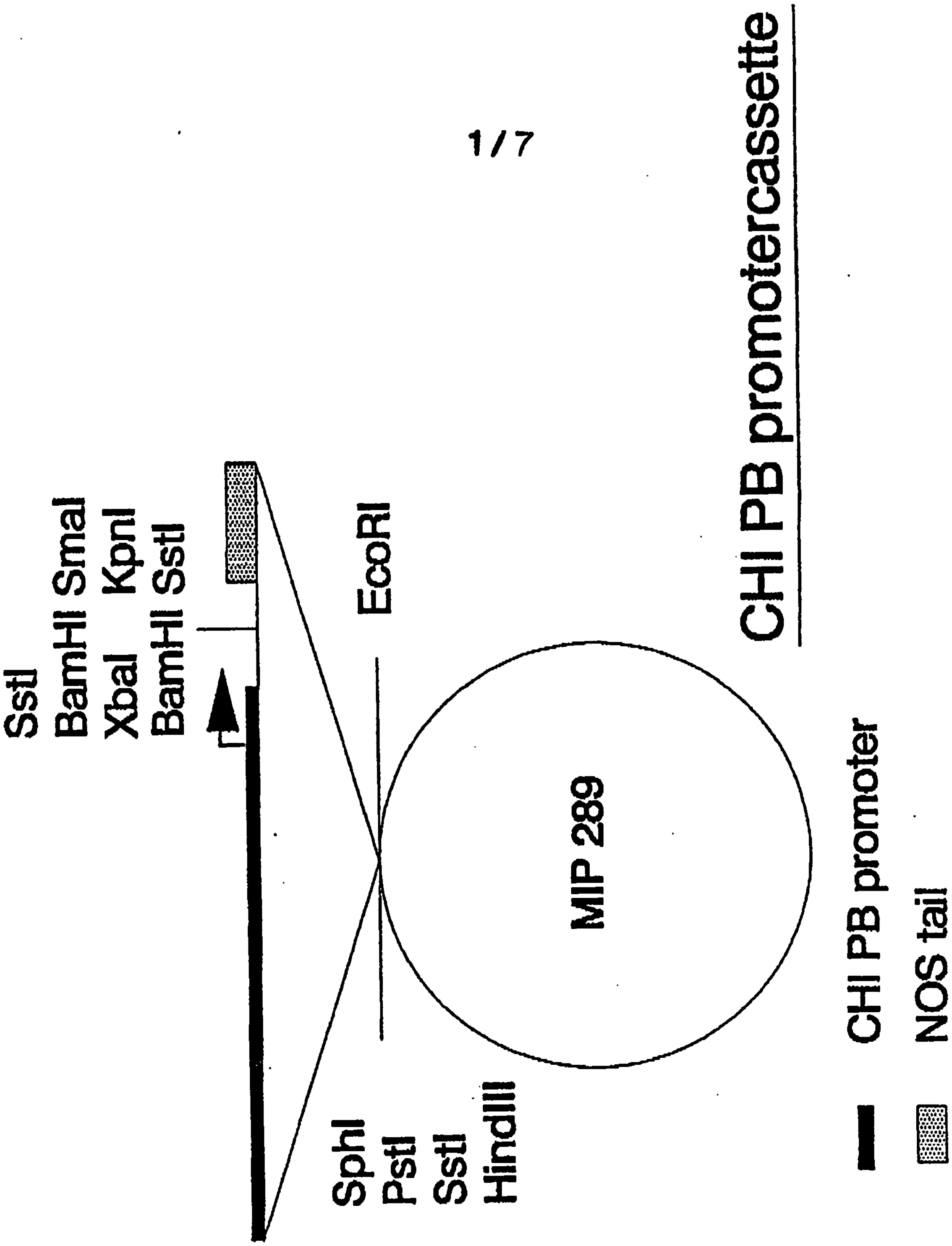

CHI PB promotercassette
SstI
BamHI SmaI
XbaI KpnI
BamHI SstI
EcoRI
MIP 289
SphI
PstI
SstI
HindIII
CHI PB promoter
NOS tail

Figure 1

35S CaMV/AB promoter cassette

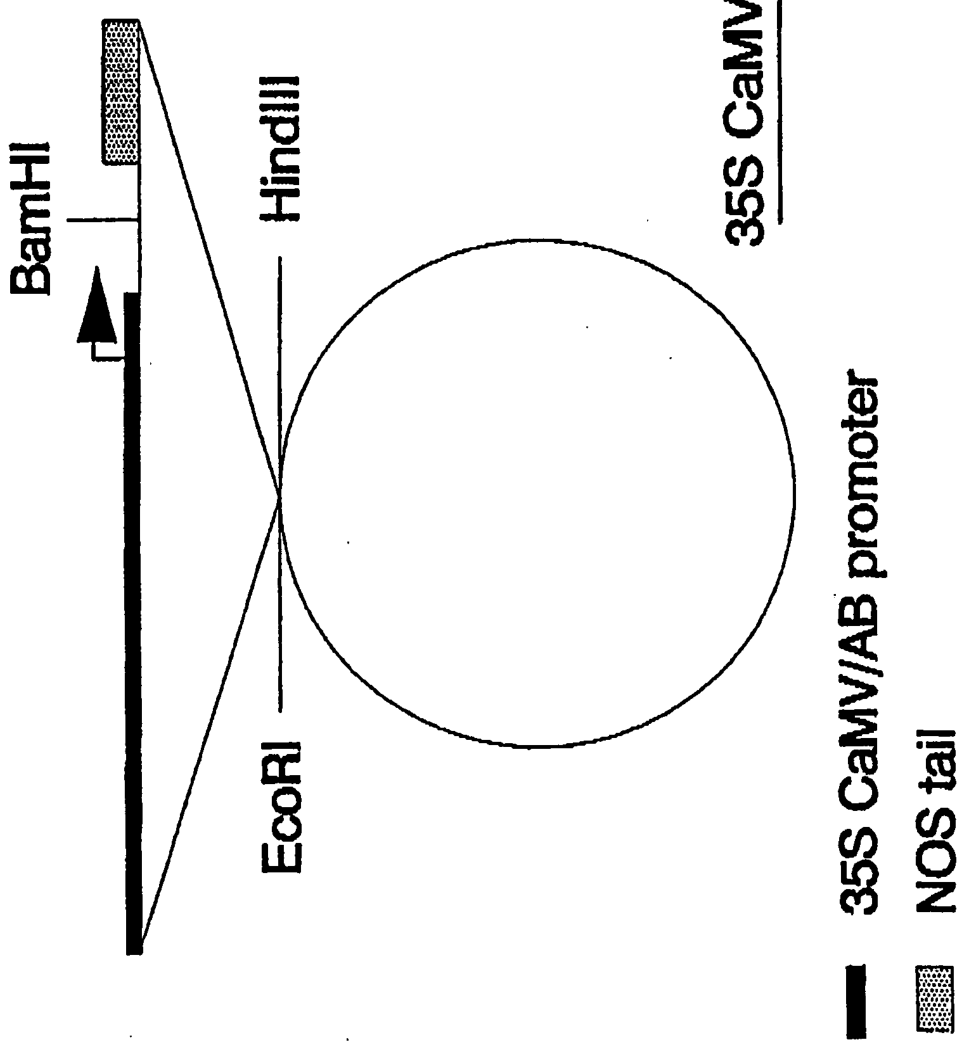


Figure 2

Ssrr X ssRr

↓

Ssrr	SsRr	ssRr	ssrr
sterile	fertile	fertile	fertile

Figure 3

Ssrr X ssRR

↓

SsRr	ssRr
fertile	fertile

Figure 4

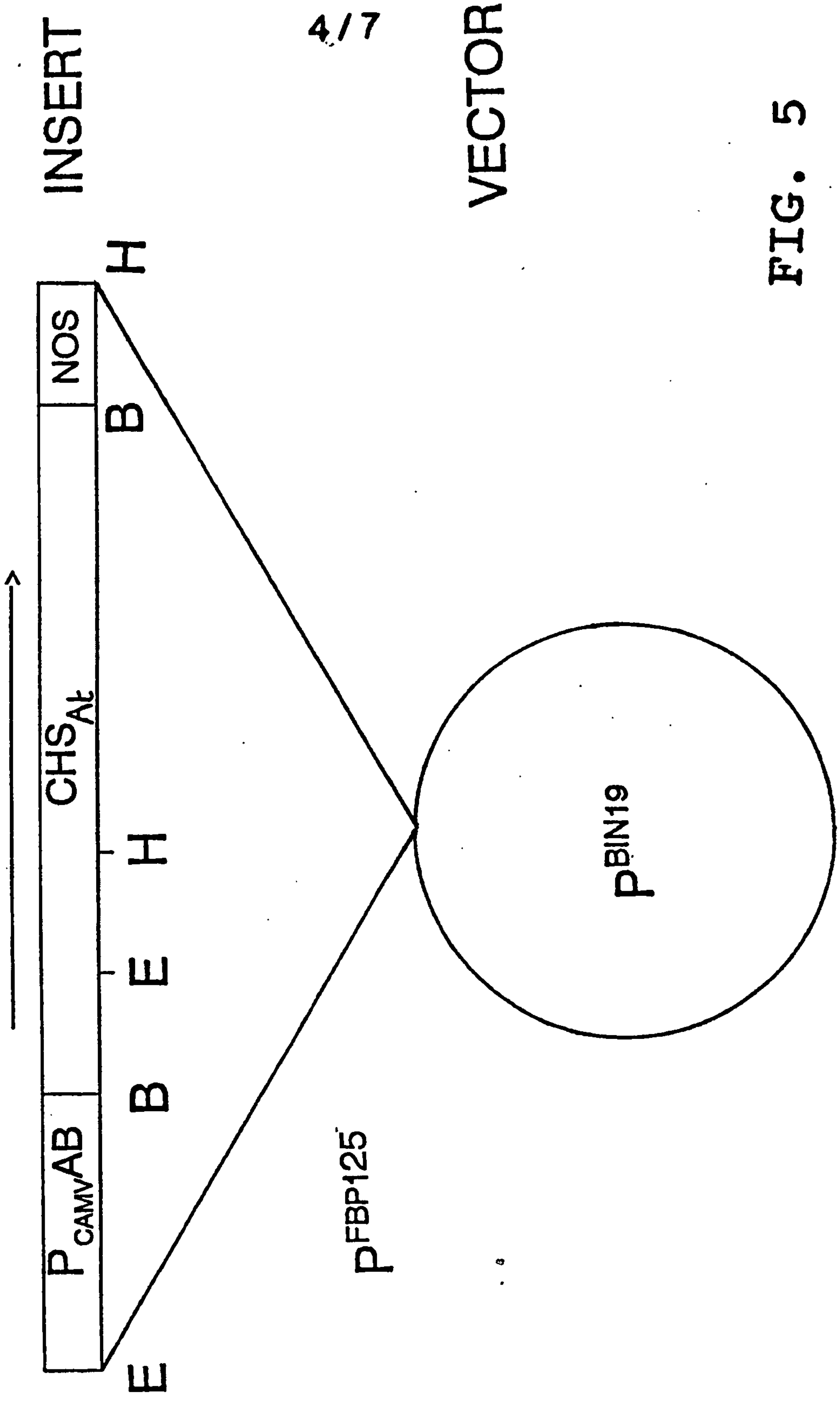


FIG. 5

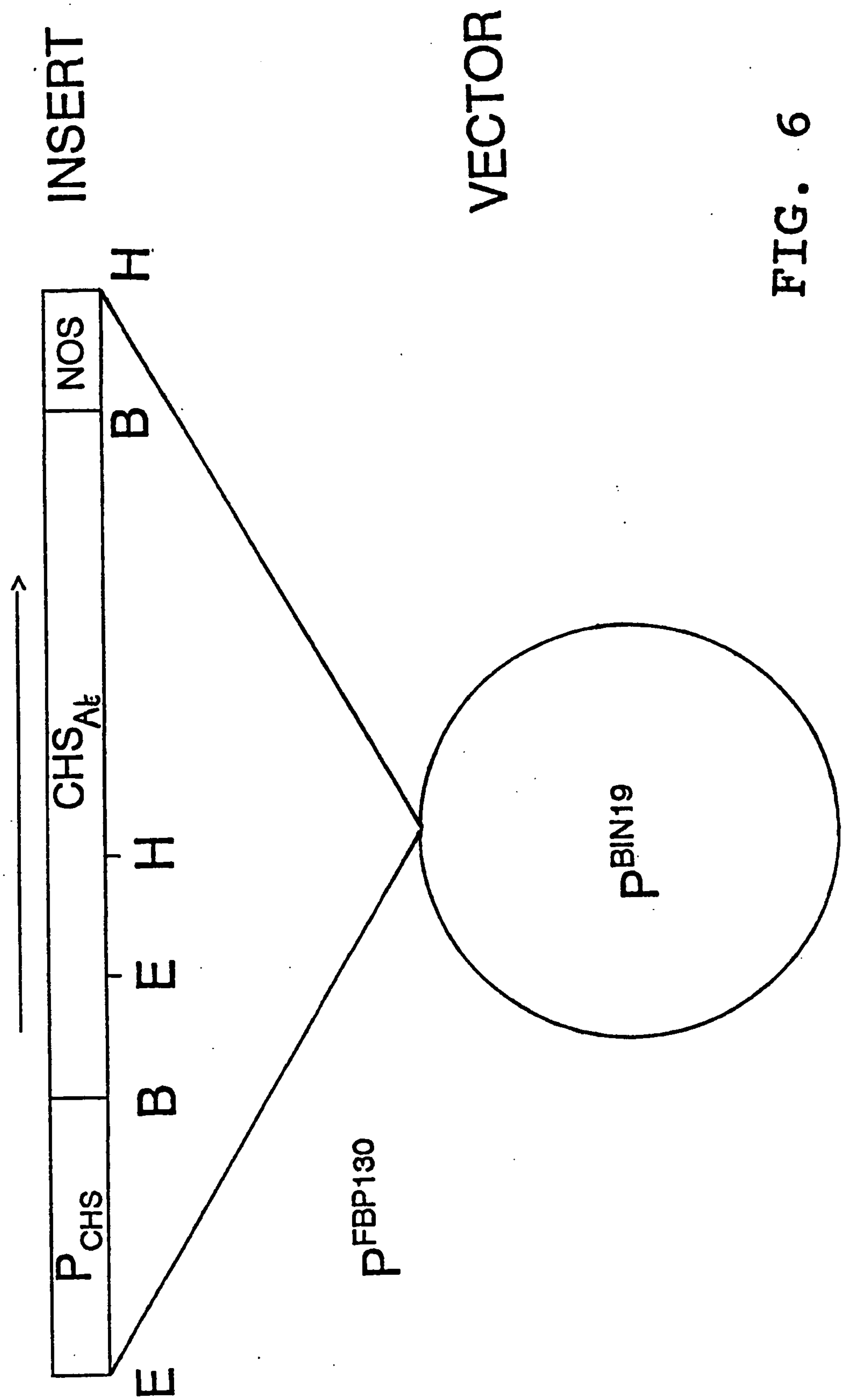
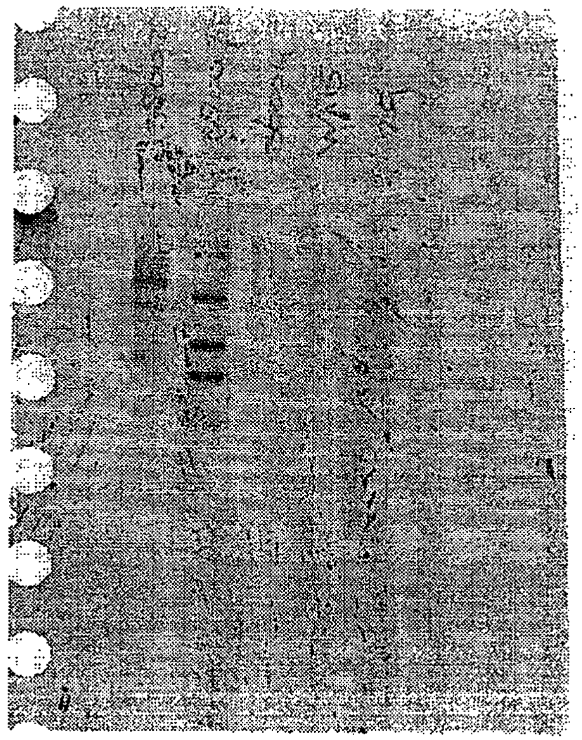


FIG. 6



Ara CHS o/n -80°, 0.1 x SSC

Figure 7.

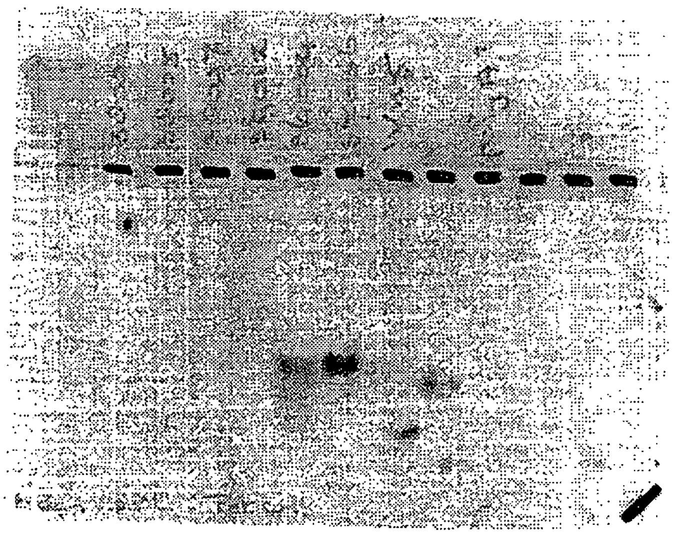


Figure 8

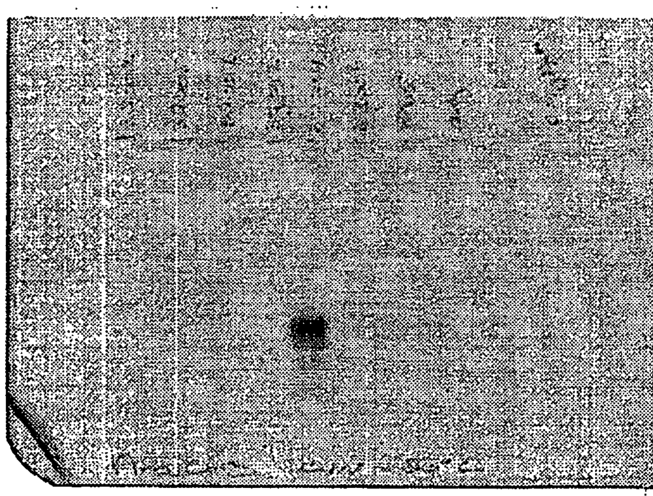


Figure 9.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

REC'D 13 JAN 1994
WIPO PCT

INTERNATIONAL FORM

Mogen International N.V.
Einsteinweg 97
2333 CB LEIDEN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
E. coli JM101 containing pFBP125	CBS 543.93

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary accepts the microorganism identified under I above, which was received by it on Thursday, 14 October 1993 *(date of the original deposit)*[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on not applicable *(date of the original deposit)* and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable *(date of receipt of request for conversion)*

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): drs F.M. van Asma Date: Friday, 19 November 1993

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V. Einsteinweg 97 2333 CB LEIDEN Nederland *name and address of the party to whom the viability statement is issued*	VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Mogen International N.V. Address: Einsteinweg 97 2333 CB LEIDEN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 544.93 Date of the deposit or of the transfer:[1] Thursday, 14 October 1993

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on Friday, 15 October 1993 [2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): drs F.M. van Asma Date: Friday, 19 November 1993

[4] Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V.
Einsteinweg 97
2333 CB LEIDEN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
E. coli JM101 containing pFBP130	CBS 544.93

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary accepts the microorganism identified under I above, which was received by it on Thursday, 14 October 1993 *(date of the original deposit)*[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on not applicable *(date of the original deposit)* and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable *(date of receipt of request for conversion)*

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): drs F.M. van Asma Date: Friday, 19 November 1993

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V. Einsteinweg 97 2333 CB LEIDEN Nederland
name and address of the party to whom the viability statement is issued

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Mogen International N.V. Address: Einsteinweg 97 2333 CB LEIDEN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 543.93 Date of the deposit or of the transfer:[1] Thursday, 14 October 1993

III. VIABILITY STATEMENT
The viability of the microorganism identified under II above was tested on Friday, 15 October 1993 [2]. On that date, the said microorganism was [X][3] viable [ ][3] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): drs F.M. van Asma Date: Friday, 19 November 1993

[4] Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02875

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/11 C12N15/54 A01H1/02 A01H5/00
A01H5/10 C12N9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE vol. 330 , 17 December 1987 , LONDON GB pages 677 - 678 MEYER, P., ET AL. 'A new petunia flower colour generated by transformation of a mutant with a maize gene' see the whole document ---	21-23
X	PLANT MOLECULAR BIOLOGY. vol. 18, no. 2 , January 1992 , DORDRECHT, THE NETHERLANDS. pages 363 - 375 DORBE, M.-F., ET AL. 'The tomato nia gene complements a Nicotiana plumbaginifolia nitrate reductase-deficient mutant and is properly regulated' see the whole document --- -/--	21-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 1994	21 -03- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 08828 (PALADIN HYBRIDS) 9 August 1990	1,6
Y	see page 16, line 4 - line 20	11-15, 19,20
	see page 101, line 1 - line 30	
P,X	WO,A,93 10251 (MOGEN) 27 May 1993	1-5, 21-23
	see page 15, line 27 - page 17, line 2 see page 55, line 15 - page 58, line 30	
P,X	WO,A,93 02197 (NICKERSON BIOCEM) 4 February 1993 see page 36, line 25 - page 37, line 8	1,6
P,X	EP,A,0 513 884 (MOGEN) 19 November 1992 see page 8, line 16 - line 27	1,6
Y	THE PLANT CELL. vol. 4, no. 3 , March 1992 , ROCKVILLE, MD, USA. pages 253 - 262 VAN DER MEER, I.M., ET AL. 'Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility' see the whole document	11-15, 19,20
Y	EP,A,0 412 911 (PLANT GENETIC SYSTEMS) 13 February 1991 see the whole document	11-15, 19,20
A	BIOTECHNOLOGY vol. 8, no. 5 , May 1990 , NEW YORK US pages 459 - 464 ROBERT, L.S., ET AL. 'Antisense RNA inhibition of beta-glucuronidase gene expression in transgenic tobacco can be transiently overcome using a heat-inducible beta-glucuronidase gene construct' see the whole document	1-23

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 93/02875

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9008828	09-08-90	AU-A-	5037290	24-08-90
		EP-A-	0456706	21-11-91
		JP-T-	4504355	06-08-92
WO-A-9310251	27-05-93	AU-A-	2928492	15-06-93
WO-A-9302197	04-02-93	AU-A-	2361492	23-02-93
EP-A-0513884	19-11-92	AU-A-	1698992	17-11-92
		WO-A-	9218625	29-10-92
EP-A-0412911	13-02-91	AU-B-	625509	16-07-92
		AU-A-	6068890	11-03-91
		WO-A-	9102069	21-02-91
		JP-T-	3503004	11-07-91